Hydrocortisone Reduces Both Constitutive and UV-Elicited Release of Epidermal Thymocyte Activating Factor (ETAF) by Cultured Keratinocytes

Thomas S. Kupper, M.D. and Joseph McGuire, M.D.

Departments of Surgery, Pathology (TSK), and Dermatology (JM, TSK), Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut, U.S.A.

Epidermal thymocyte activating factor (ETAF) is spontaneously released into the media by PAM 212 and A 431 cell lines and cultured normal human keratinocytes. ETAF from all 3 cell types can substitute for interleukin 1 (IL-1) in the augmentation of proliferation of a helper T-cell clone (D10.G4.1) induced by mitogen. Hydrocortisone (HC) substantially reduces the release of ETAF by these keratinocytes and, further, appears to induce the release of an inhibitor of lymphocyte activating factor activity of IL-1. Irradiation with UVC causes increased ETAF release into the media. Hydrocortisone abrogates this effect. Thus HC reduces both constitutive and elicited release of ETAF. ETAF plays a major role in inflammation; the ability of HC to block ETAF release by keratinocytes may account for the anti-inflammatory effect of glucocorticosteroids on the skin.
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Interleukin 1 (IL-1), a polypeptide released by cells of the monocyte and macrophage lineage, mediates a wide variety of inflammatory responses [1,2]. Keratinocytes produce a cytokine which is biochemically and functionally similar to IL-1 and is termed epidermal cell-derived thymocyte activating factor or ETAF [3,4]. Like IL-1, ETAF is a lymphocyte activating factor (LAF), a pyrogen, an inducer of acute phase reactant synthesis, a mitogen for fibroblasts, and is chemotactic for neutrophils and T cells [1–6]. The discovery that an IL-1-like molecule is produced by keratinocytes, together with the evidence that IL-1 has an important role in inflammation, has led to speculation concerning the role of ETAF in cutaneous inflammation.

We examined the regulation of ETAF release from cultured epidermal cells, including PAM 212, a spontaneously transformed mouse keratinocyte cell line [7], and A 431, a cell line derived from a human vulvar carcinoma [8], both of which had already been shown to release ETAF into the media. Long-term cultures of neonatal human foreskin keratinocytes (HFEC) were also examined. In these studies, we examined the LAF activity of ETAF. The assay most frequently used for LAF activity is the murine thymocyte costimulator assay [9]. We have chosen a more sensitive assay for LAF activity in which a cloned T helper lymphocyte, D10.G4.1, is the responder cell [10].

In this study we demonstrate that hydrocortisone (HC) reduces ETAF release from cultured keratinocytes. We propose that this effect on ETAF may underlie the anti-inflammatory effects of glucocorticosteroids (GCS) on skin.

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Reprint requests to: Thomas S. Kupper, M.D., Howard Hughes Medical Institute, Department of Pathology, Yale University School of Medicine, 310 Cedar Street, New Haven, Connecticut 06510.

Abbreviations:
CM: conditioned medium (-ia)
Con A: concanavalin A
ETAF: epidermal cell-derived thymocyte activating factor
GCS: glucocorticosteroid(s)
HC: hydrocortisone
HFEC: human foreskin epidermal cells (keratinocytes)
HPLC: high-performance liquid chromatography
IL-1: interleukin 1
LAF: lymphocyte activating factor
WBC: white blood cell(s)

MATERIALS AND METHODS

Chemicals Hydrocortisone and concanavalin A (Con A) were obtained from Sigma Chemical Company (St. Louis, Missouri).

Cell Cultures PAM 212, a spontaneously transformed keratinocyte line from BALB/c mice, was provided by Dr. Pamela Hawley-Nelson (National Cancer Institute). A 431, a tetraploid epidermal cell line from a vulvar carcinoma, was provided by Dr. Lloyd King (Vanderbilt University). Both cell lines were maintained in Dulbecco's minimal essential medium (Gibco) with 10% calf serum. Confluent cultures were used for experiments.

Neonatal human foreskin was the source of normal human keratinocytes (HFEC). The cultures were initiated and maintained by the technique of Rheinwald and Green [11].

In experiments which measured the effect of HC on ETAF production, cultures were grown in the presence or absence of HC under *serum-free* conditions. Media collected from these cultures were extensively dialyzed against Cantor's balanced salt solution.

D10 Assay for Interleukin 1 D10.G4.1 (D10), a murine helper T-cell clone, and 3D3, a monoclonal clonotype-specific antibody which activates the T-cell receptor of D10, were provided by C. Janeway, Jr. (Yale University). D10 cells were maintained as previously described [10].

Measurement of IL-1 activity utilizes D10 as a responder cell and either Con A or the clonotype-specific monoclonal antibody 3D3 as costimulus, as described by Kaye and Janeway [10]. Briefly, 2×10^4 D10 cells, a 1:1000 dilution of 3D3 hybridoma supernatant, or 2.5 μg/ml of Con A, and the experimental sample were cocultured in triplicate in a final volume of 200 μl in Costar 96-

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well tissue plates. Clicks medium with 5% fetal calf serum was used and cultures were incubated for 72 h at 37°C in a 5% CO_2/95% air incubator. Six hours before harvest, 1 μCi/well of [^{3}H]thymidine was added. Cultures were harvested on a MASH (Cambridge Technologies) and counted in a Beckman scintillation spectrometer.

UVC Irradiation Keratinocyte cultures in serum-free media were irradiated with 0.1 J/cm^2. Fresh medium was then added and cultures were incubated for another 24 h. Medium was then removed and dialyzed against phosphate-buffered saline.

RESULTS

Previous studies in our laboratory have shown that media conditioned by HFEC contain less than 5% of ETAF/LAF activity of media conditioned by PAM 212 and A 431 (unpublished). Hydrocortisone 10^{-6} is routinely included in the media of human keratinocyte cultures [11], but is not present in PAM 212 and A 431 media. When HFEC were cultured in the absence of HC, ETAF activity in the medium was increased to levels equivalent to those found in PAM- and A 431-conditioned media (CM).

GCS directly inhibit T-lymphocyte proliferation, in part by inhibiting transcription of the IL-2 gene [12]. Since LAF activity is thought to depend on the synthesis of IL-2 by T cells in response to IL-1 and mitogen [13], it was not surprising that undialyzed CM containing HC inhibited D10 proliferation in the IL-1 assay. When D10 proliferation in response to ETAF/IL-1 and Con A was measured in the presence of varying amounts of HC, the inhibitory effect of HC was clearly dose dependent. HC at a final concentration of 2.5×10^{-6} M completely inhibited proliferation of D10; final concentrations of 2.5×10^{-8} M HC or less were not inhibitory (data not shown). It was determined that dialysis effectively removed HC in serum-free CM so that final concentrations of HC, as tested by radioimmunoassay, were less than 2.5×10^{-8} M. In the present study, CM was never used at greater than a 25% (vol/vol) concentration in the D10 assay. Therefore, direct inhibition of D10 by HC was not observed when *dialyzed* serum-free media were tested.

To determine whether HC inhibited ETAF release by keratinocytes, HFEC and PAM 212 were grown under serum-free conditions in the absence of HC or in the presence of 10^{-6} and 10^{-5} M HC. Fig 1 (HFEC) and Fig 2 (PAM 212) show that ETAF/LAF activity in media from these cell lines is significant when they were cultured in the absence of HC for 10 days. ETAF/LAF activity in CM was progressively reduced when cells were grown in the presence of 10^{-6} M or 10^{-5} M HC over the same time period. This effect was more striking on PAM 212 cultures; in the presence of 10^{-5} M HC, ETAF release by PAM 212 is completely blocked. Cell viability and morphology, however, were similar in all groups under all of the above conditions. Furthermore, total protein was not diminished in the cultures by 10^{-5} M HC (data not shown). These experiments indicated that release of ETAF into the media was inhibited by HC.

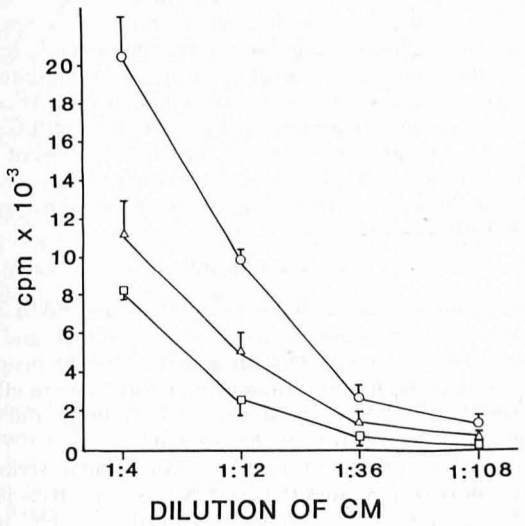

Figure 1. Reduction of HFEC, ETAF by HC. HFEC was cultured under serum-free conditions for 10 days in either 10^{-6} M (*triangles*), 10^{-5} M (*squares*), or no (*circles*) HC. All CM were dialyzed prior to incubation with D10. Serial dilutions of CM were mixed with 2×10^4 D10 cells in Clicks medium with 5% fetal calf serum to a final concentration of 25% by volume. 3D3 was used at a final concentration of 1:1000.

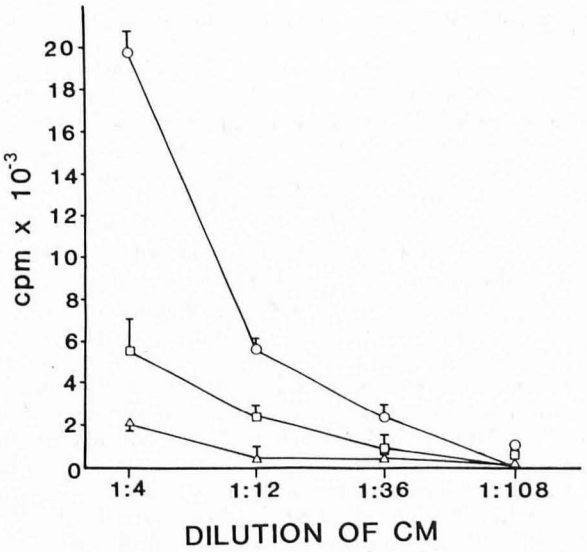

Figure 2. Reduction of PAM 212 ETAF by HC. Conditions are identical to Fig 1. No HC (*circles*), 10^{-6} M HC (*squares*), 10^{-5} M HC (*triangles*).

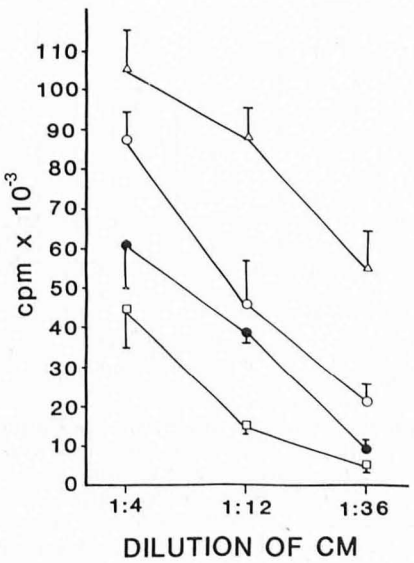

Figure 3. Reduction of constitutive and UV-elicited ETAF by HC in HFEC media. HFEC were grown in the presence (10^{-6}) and absence of HC for 72 h under serum-free conditions. At this time, some cultures received 0.1 J/cm^2 of UVC (254 mn). Fresh media with or without 10^{-6} M HC were added to all cultures and removed 24 h later. These media were dialyzed extensively before inclusion in the D10 assay. Conditions of the D10 assay are those outlined in Figs 1 and 2. Groups are: no HC (○—○); no HC, UVC (△—△); 10^{-6} M HC (□—□); and 10^{-6} M HC, UVC (●—●).

UV irradiation has been shown to enhance ETAF release by keratinocytes [14]. We asked whether HC influenced the amount of ETAF released after UV irradiation. Spontaneous ETAF release from HFEC is significantly reduced by 10^{-6} M HC (Fig 3). Furthermore, while cells irradiated in the absence of HC released significantly more ETAF than unirradiated cells, cells irradiated in the presence of HC released significantly less additional ETAF. Thus, both constitutive and elicited ETAF release are inhibited by HC.

Urine, serum, and media from certain cell cultures contain inhibitors of LAF activity [15–17]. Therefore, the possibility that keratinocytes grown in HC produced an inhibitor of ETAF/LAF activity was tested. Media from cells grown in the presence of 10^{-5} M HC still inhibited ETAF activity even after dialysis, which reduced HC to levels not inhibitory to D10 (as determined by radioimmunoassay). This finding suggested the presence of a non-dialyzable inhibitor. CM from all 3 cell lines contained an inhibitor(s) which decreased proliferation of D10 in response to ETAF (Fig 4*a*). This inhibition by both HFEC- and PAM-CM, while significant at a 1:4 dilution, was not seen at a 1:36 dilution (Fig 4*a*). At this dilution, however, there was still less LAF activity in media from cells grown in the presence of HC than in media from cells grown in the absence of HC (Fig 4*b*). While an inhibitor may account in part for the difference in LAF activity at 1:4 dilutions, the difference in LAF activity between cells grown in the presence or absence of HC at the 1:36 dilution cannot be attributed to the presence of inhibitor(s) alone. CM from A 431 cells cultured with HC contained very high levels of inhibitory activity which was significant even at a 1:160 dilution. Since little LAF activity was demonstrable in A 431 grown with HC even at such dilutions, the relative contribution of inhibitor vs diminished ETAF release to LAF activity could not be determined for this cell line.

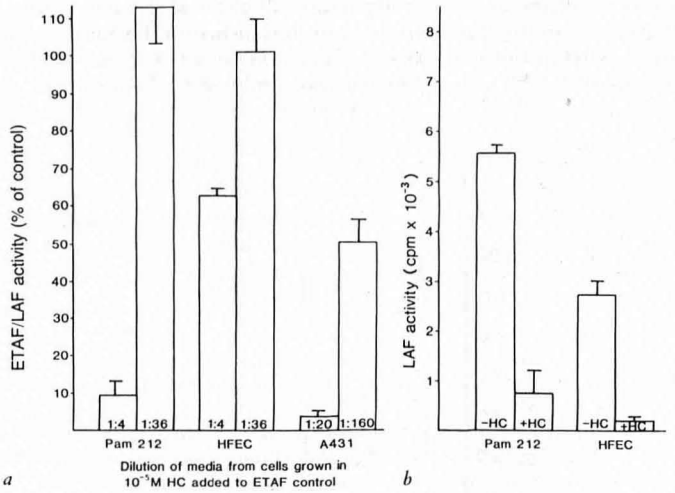

Figure 4. *a,* Inhibition of ETAF/LAF activity by media from keratinocytes grown in the presence of HC. PAM 212, HFEC, and A 431 cells were grown in Dulbecco's minimal essential medium (10^{-5} M HC) without serum for 10 days. Media were then collected and dialyzed. Inhibition of LAF activity by dialyzed media from cells grown in the presence of HC was tested as follows: serum-free CM from either PAM, HFEC, or A 431 cells grown in the *absence* of HC and containing 50 U/ml of LAF activity was added to D10 and Con A as previously described. In addition to this ETAF control, serial dilutions of dialyzed media from the corresponding cells grown in the presence of 10^{-5} M HC were added to the cultures. *b,* Reduced LAF activity in media from cells grown in the HC reflects reduced ETAF release. Both PAM 212 and HFEC cells were grown for 10 days in the presence or absence of 10^{-5} M HC. These media were dialyzed and tested for LAF activity at 1:36 dilution, a dilution at which no inhibitory activity from PAM 212 and HFEC medium could be demonstrated in the D10 assay (see *a*). These results demonstrate that there is significantly less LAF activity in media from cells grown in 10^{-5} M HC independent of the effect of HC and the HC-induced inhibitor or the D10 assay.

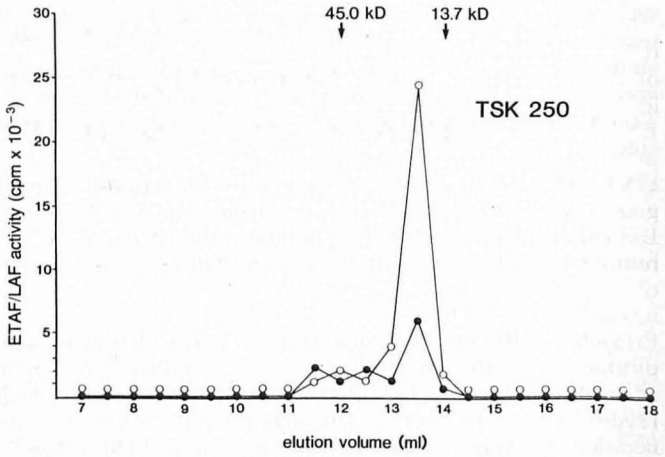

Figure 5. Gel filtration HPLC analysis of media from HFEC grown under serum-free conditions in the presence or absence of HC. Media from HFEC grown for 10 days in 10^{-5} M HC (*solid circles*) or in its absence (*open circles*) were dialyzed [10] and tested for LAF activity in the D10 assay. Media from cells grown in the absence of HC contained 36 units/ml and media from cells grown in 10^{-5} M HC contained 8 units/ml. A volume of 0.5 ml was analyzed by HPLC using a Biosil TSK 250 column. The flow rate of the mobile phase (PBS) was 1 ml/min. Twenty-four fractions of 0.5 ml each were collected, filtered, diluted 1:2 in tissue culture medium, and tested at a 1:4 dilution in the D10 assay. Molecular weight standards were run separately.

The observation that an inhibitor was present in the media of cells grown in HC raised 3 possibilities: (1) HC does not affect ETAF release; decreased ETAF activity is due to the presence of an inhibitor. (2) HC reduces ETAF release in addition to generating an inhibitor of ETAF activity. (3) By reducing ETAF release, HC reveals the presence of a constitutive inhibitor which is not influenced by altering HC concentration. In order to test the first 2 possibilities, medium from HFEC grown in the presence or absence of 10^{-5} M HC was analyzed by gel filtration high-performance liquid chromatography (HPLC) using a TSK-250 column. All of the ETAF/LAF activity from cells grown in the absence of HC elutes in a single fraction (Fig 5) between 15–20 kD. Media from cells grown in HC contain significantly less LAF activity in this fraction. None of these fractions inhibited D10 proliferation, thus eliminating the possibility that ETAF and the inhibitor coelute and suggesting that gel filtration HPLC of unconditioned media probably reduced the concentration of the inhibitor to below detectable levels. Preliminary evidence, from studies using the A 431 cell line, suggest that the inhibitor is larger than 50 kD (data not shown).

DISCUSSION

These observations indicate that in both HFEC and PAM 212 cell lines, HC effects both diminished release of ETAF and either reveals or induces a factor which antagonizes its LAF properties. It is not known whether this inhibitory factor(s) has an effect on other activities of ETAF (e.g., fibroblast activation), and its relevance in vivo remains to be demonstrated.

HC is normally present at 5×10^{-7} M in human serum, but may reach more than 3 times this level with severe stress [18,19]. It may be physiologically significant that 10^{-6} M HC inhibits ETAF release by both human and murine keratinocytes. Conversely, cells grown in the absence of HC spontaneously release large amounts of ETAF into the culture medium. On the basis of this relationship between HC and ETAF release, it can be speculated that release of ETAF in the epidermis may correlate inversely with the concentration of HC in tissue.

Inflammatory disorders of the skin are often treated with corticosteroids. The observation that pharmacologic concentrations of HC (10^{-5} M) more completely inhibit ETAF release than physiologic levels suggests a mechanism by which GCS may modify ETAF-induced features of the inflammatory response: recruitment of neutrophils and mononuclear cells from the systemic circulation, stimulation of fibroblast proliferation and prostaglandin production, and activation of T lymphocytes [1–6]. Since ETAF has been linked to the initiation and maintenance of wound healing, it can be speculated that the profound inhibitory effects of high levels of GCS on wound healing may result in part from impaired ETAF release at the wound.

In 1970 cortisol was shown to reduce the amount of endogenous pyrogen released from human white blood cells (WBC) preparations in response to phagocytosis of heat-killed staphylococci [20]. This observation may now be interpreted as a cortisol-mediated reduction in the release of IL-1 from macrophages present in the WBC preparation. More recently, media conditioned by both murine [21] and rat [22] peritoneal exudate cells stimulated with lipopolysaccharide or carageenan were shown to contain less LAF activity in the presence of HC. It is notable that 10^{-6} M HC was effective in blocking IL-1 release from macrophages in these studies. However, while suggesting a relationship between corticosteroids and IL-1 release, these studies were performed on heterogenous cell populations and could not conclusively prove that the effect of corticosteroids on macrophages was not mediated by a nonmacrophage cell type. The use of keratinocyte cell lines and long-term cultures of human keratinocytes in this report indicates that the effect of corticosteroids on ETAF release by keratinocytes does not require the presence of another cell type.

The mechanism by which HC regulates ETAF release cannot be determined from the present study. Many steroids exert their effect by regulating gene transcription [23,24]. It is possible that transcription of the ETAF/IL-1 gene is inhibited by HC; it is also possible that HC regulates another gene product which influences the release of ETAF from the keratinocyte. The use of cDNA probes for both murine [25] and human [26] IL-1 will determine whether corticosteroids regulate ETAF/IL-1 gene expression, and these studies are currently under way in our laboratory.

In summary, cultures of A 431, PAM 212, and HFEC all spontaneously release ETAF into the media. From all 3 keratinocyte cell types, ETAF can substitute for IL-1 in the augmentation of proliferation of D10 cells induced by mitogen or by activation of the T-cell receptor by a monoclonal antibody. Growth of these keratinocytes, in the presence of physiologic or pharmacologic concentrations of HC, results in a significant reduction of ETAF/LAF activity. This occurs by 2 mechanisms: reduction of the release of ETAF by keratinocytes and the release of an inhibitor of LAF activity.

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